Peripheral microRNA panels to guide the diagnosis of familial cardiomyopathy

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Etiology-based diagnosis of dilated cardiomyopathy (DCM) is challenging. We evaluated whether peripheral microRNAs (miRNAs) could be used to characterize the DCM etiology. We investigated the miRNA plasma profiles of 254 subjects that comprised 5 groups: Healthy subjects (n = 70), idiopathic DCM patients (n = 55), ischemic DCM patients (n = 60) and 2 groups of patients with pathogenic variants responsible for familial DCM in the *LMNA* (*LMNA*^{MUT}, n = 37) and *BAG3* (*BAG3*^{MUT}, n = 32) genes. Diagnostic performance was assessed using receiver operating characteristic curves. In a screening study (n = 30), 179 miRNAs robustly detected in plasma samples were profiled in idiopathic DCM and carriers of pathogenic variants. After filtering, 26 miRNA candidates were selected for subsequent quantification in the whole study population. In the validation study, a 6-miRNA panel identified familial DCM with an AUC (95% confidence interval (CI)) of 87.8 (82.0–93.6). The 6-miRNA panel also distinguished between specific DCM etiologies with AUCs ranging from 85.9 to 89.9. Only 1 to 10 of the subjects in the first and second tertiles of the 6-miRNA panel were patients with familial DCM. Additionally, a 5-miRNA panel showed an AUC (95% CI) of 87.5 (80.4–94.6) for the identification of carriers with pathogenic variants who were phenotypically negative for DCM. The 5-miRNA panel discriminated between carriers and healthy controls with AUCs ranging from 83.2 to 90.8. Again, only 1 to 10 of the subjects in the lowest tertiles of

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the 5-miRNA panel were carriers of pathogenic variants. In conclusion, miRNA signatures could be used to rule out patients with pathogenic variants responsible for DCM. (Translational Research 2020; 218:1−15)

Abbreviations: AUC = area under the curve; *BAG3* = BCL2-associated athanogene 3; CAD = coronary artery disease; CI = confidence interval; DCM = dilated cardiomyopathy; HF = heart failure; *LMNA* = Lamin A/C; LV = left ventricular; LVEF = LV ejection fraction; LVEDD = LV end-diastolic diameter; miRNA = microRNA; ncRNA = noncoding RNA; ROC = receiver operating characteristic

AT A GLANCE commentary

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Background

Dilated cardiomyopathy is a complex and heterogeneous disease with a common phenotype but multiple etiologies. Early recognition of the underlying etiology is essential for risk stratification. However, overlapping morpho-functional phenotypes make differential diagnosis challenging.

Translational Significance

Panels combining microRNAs could serve as biomarkers to discard the presence of pathogenic variants associated with familial dilated cardiomyopathy. Our multimarker panel could provide a novel approach for initial triage or even differential diagnosis strategies in patients with suspected familial dilated cardiomyopathy. The potential of microRNAs to discard the presence of pathogenic variants within the generally low-risk population is also promising.

INTRODUCTION

Dilated cardiomyopathy (DCM) is a clinical diagnosis defined as left ventricular (LV) or biventricular dilatation and systolic dysfunction that are not attributable to abnormal loading conditions or coronary artery disease (CAD).[1] DCM is a common form of cardiomyopathy with an estimated prevalence of up to 1:250/500 in adults.[2] This condition is the most frequent indication for heart transplantation worldwide, is one of the main causes of heart failure (HF) and is associated with marked rates of morbidity and mortality.[3,4]

DCM is a complex and heterogeneous disease with a common phenotype but multiple etiologies. In more than 70% of nonischemic cardiomyopathy patients, a clinical diagnosis of idiopathic DCM is assigned when no identifiable cause is found after etiological assessment.[2] For idiopathic DCM, a positive familial history can be identified in up to 30%−50% of cases.[5] More than 400 pathogenic variants responsible for familial DCM have been described in nearly 60 disease-related genes,[6,7] including the Lamin A/C (*LMNA*) and BCL2-associated athanogene 3 (*BAG3)* genes. Importantly, the prognosis is intimately associated with the etiology. Concerning familial DCM, the phenotype associated with *LMNA* and *BAG3* rare pathogenic variants leads to a high risk of death, HF, heart transplantation and malignant ventricular arrhythmias.[8,9] Early recognition of the underlying etiology is essential for personalized risk stratification, for example, specific treatments targeted to the underlying cause or need for genetic screening. However, the identification of the DCM etiology is a challenge. Overlapping morphofunctional phenotypes make differential diagnosis difficult. Clinically, there are no differences between familial and idiopathic DCM. Personal and family history review, physical examination, imaging, and laboratory variables assist in clinical decision-making but are insufficient to define the etiology. Clinical genetic testing is becoming time- and slightly cost-effective.[10] However, the interpretation of results could be complicated, and etiology-based diagnosis rarely begins with genetic counseling/testing. There is an urgent demand for the identification of novel biomarkers to aid clinicians in DCM care.

Transcriptome-originating biomarkers are emerging clinical indicators.[11] The transcriptomic signature, in particular, noncoding RNAs (ncRNAs), could provide insights into both the physiological and pathological states of a patient,[12] with added value to routine approaches based on genome analyses.[13] In contrast to the DNA sequence, which is constant, ncRNA expression is highly dynamic and rapidly altered in response to stressors.[14] Furthermore, ncRNA alterations could be indicative of the complex interactions between genetic and environmental factors. Comorbidities, psychosocial factors, drug treatments, and other exogenous factors not routinely considered during clinical assessment influence ncRNA expression.[15] Based on their biological characteristics, the ncRNA profile can serve as a molecular fingerprint to characterize pathological conditions. Indeed, the deregulation of the ncRNA profile is correlated with numerous cardiovascular conditions.[16]

Of the various classes of ncRNAs investigated, the most attention has been focused on microRNAs (miRNAs). miRNAs can be easily extracted from peripheral blood noninvasively or with minimal

invasiveness, and they can be quantified using technology used in clinical laboratories, such as PCR. Previous research suggests that miRNA-based testing is a cost-effective strategy in disease monitoring.[17,18] Multiple studies have proposed their utility as plausible diagnostic biomarkers.[19,20] Despite the potential clinical application of miRNAs, their use in the differential diagnosis of DCM has been poorly explored.[21] In a small hypothesis-generating study, we previously showed that a peripheral miRNA signature could assist in the diagnosis of patients with familial DCM caused by *LMNA* pathogenic variants.[22] Here, we complete and extend our previous findings by assessing the utility of peripheral miRNAs as biomarkers for DCM management.

MATERIAL AND METHODS

Study population and study design. This was an observational, case-control, and multicentric study. The study design is shown in Fig 1. A total of 254

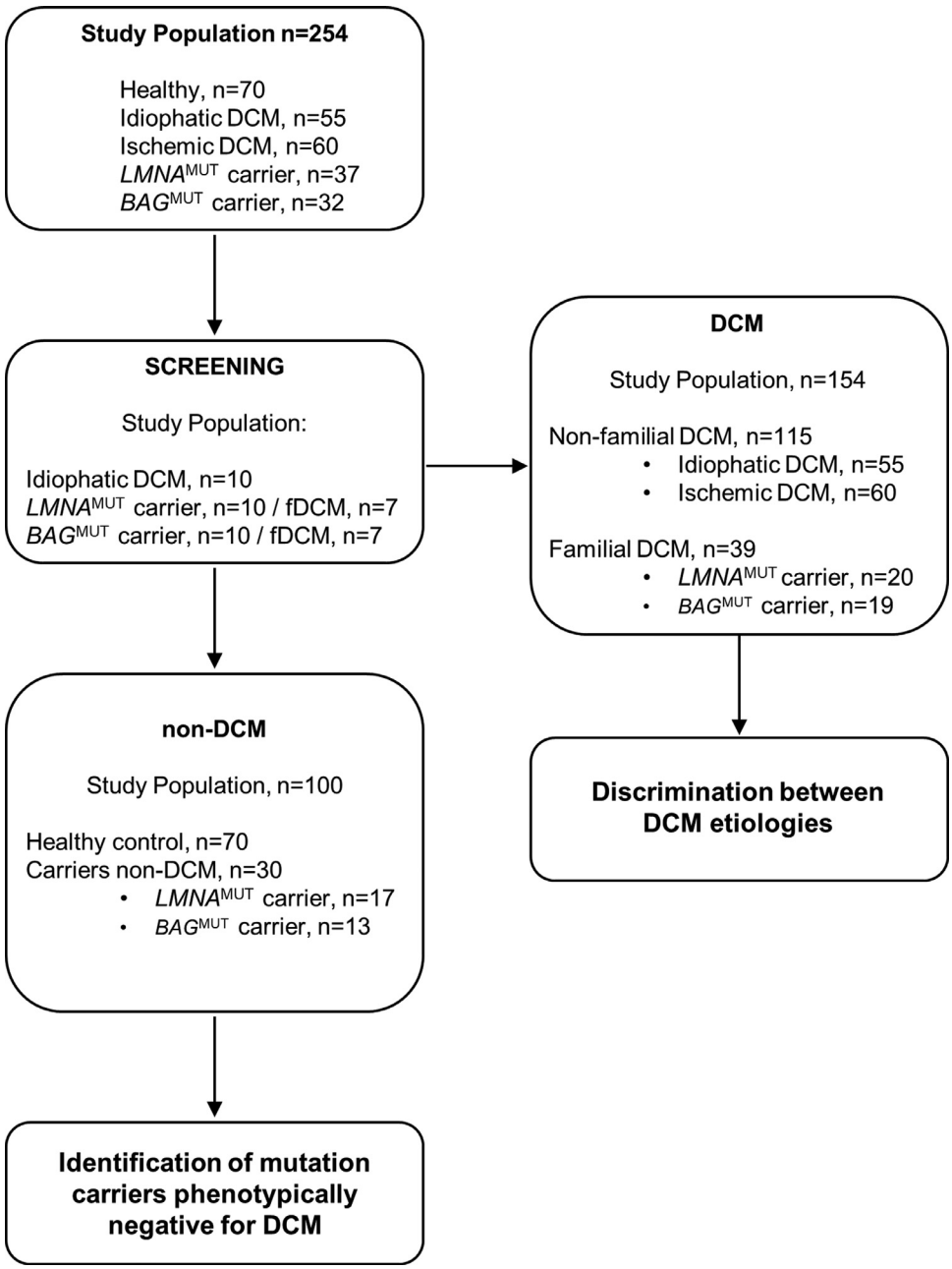

Fig 1. Study design.

participants were enrolled (Table 1). The study population included 5 study groups: Healthy controls (n = 70), idiopathic DCM patients (n = 55), ischemic DCM patients (n = 60), and 2 groups of carriers of rare pathogenic variants in the *LMNA* gene (*LMNA*^{MUT}, n = 37) and *BAG3* gene (*BAG3*^{MUT}, n = 32) that are responsible for familial DCM. Among the groups of carriers, a total of 20 *LMNA*^{MUT} and 19 *BAG3*^{MUT} participants were phenotypically positive for the disease; fulfilled the diagnostic clinical criteria for DCM. Idiopathic DCM patients did not fulfill the familial DCM criteria. DCM etiology was adjudicated by clinical cardiologists. The participants were recruited from 5 field centers (Virgen del Rocio Universitary Hospital, Sevilla; Puerta del Mar University Hospital, Cádiz; General University Hospital Consortium of Valencia, Valencia; Cruz Roja Hospital, Madrid; and Puerta de Hierro Universitary Hospital, Madrid, Spain). DCM was defined as either LV ejection fraction (LVEF) levels less than 45% and/or LV end-diastolic diameter (LVEDD) larger than 56 mm. The information collected included clinical and medical treatment data from electronic medical records, standard questionnaires, electrocardiogram, transthoracic echocardiography and, if necessary, magnetic resonance imaging. None of the patients were under heparin therapy.

Ethics. The study protocol was approved by the respective ethics committee. The study was performed in full compliance with the Declaration of Helsinki. All participants provided written informed consent.

Genetic analysis. All patients included in our study were analyzed using NGS panels, including different number of genes associated with DCM. Despite this fact, at least *LMNA* and *BAG3* were comprehensively analyzed. Allelic frequency of genetic variants identified was consulted in Genome Aggregation Database -gnomAD- (https://gnomad.broadinstitute.org/). In addition, genetic variations were also consulted in the Human Gene Mutation Database -HGMD- (http://www.biobase-international.com/product/hgmd) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). Subjects were considered positive when a pathogenic or likely pathogenic variants in the genes *LMNA* or *BAG3* was identified, and the cosegregation of this rare variant was observed among the direct line relatives clinically diagnosed with DCM. Pathogenic or likely pathogenic variants in *LMNA* or *BAG3* genes (Supplemental Table S1) were classified according to current ACMG recommendations.[23]

Peripheral miRNA quantification. ***Blood collection.*** Ten milliliters of peripheral blood were collected in EDTA tubes (BD) in the early morning and after 10−14 h of overnight fasting. Blood samples were immediately centrifuged (1500 x g, 15 minutes, 4°C). The top layer containing plasma was divided into aliquots and stored at −80°C until further analysis. For peripheral miRNA measurements, frozen plasma aliquots were shipped on dry ice to the Biomedical Research Institute, Sant Pau (Barcelona, Spain).

RNA isolation. Total RNA was isolated from 200 μL of frozen plasma samples using the miRNeasy Serum/Plasma Advanced Kit (Qiagen) according to the manufacturer's instructions. To control for variability in RNA isolation and ensure reproducible RNA yields, a mix containing 3.5 μL of synthetic *Caenorhabditis elegans* miR-39-3p (cel-miR-39-3p) (1.6×10^8 copies/μL) and 1.5 μL of *bacteriophage* MS2 carrier RNA (Roche) was added to each sample at the beginning of the isolation. RNA was eluted into 20 μL of nuclease-free water and stored at −80°C.

Reverse transcription and quantitative PCR. Reverse transcription (RT) reactions were performed using the miRCURY LNA RT Kit (Qiagen) in a total reaction volume of 10 μL. The RT reactions were incubated for 60 minutes at 42°C, 5 minutes at 95°C, and then held at 4°C. Then, cDNA was stored at −20°C. Quantitative PCR (qPCR) was performed with the miRCURY LNA SYBR Green PCR Kit (Qiagen) in a total volume of 10 μL. qPCR was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems) at 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, and 56°C for 60 seconds, followed by melting curve analysis. For the screening study, we used the miRCURY LNA miRNA Serum/Plasma Focus PCR Panel (Qiagen). The panel included 179 miRNA primer sets previously described in human serum/plasma. For the validation study, each selected miRNA candidate was quantified in custom 384-well Pick-&-Mix microRNA PCR plates (Qiagen). The interplate calibrator UniSp3 was analyzed to account for the variability between the plates. qPCR amplification curves were evaluated with SDS v2.3 software (Applied Biosystems). The specificity of the amplification was corroborated by melting curve analysis. Cqs above 35 cycles were considered undetectable and were censored at the minimum level observed for each miRNA. cel-miR-39-3p levels were stable across all samples: median Cq (first quartile − third quartile) = 27.0 (26.0−27.8). Relative quantification was performed using the 2^{-dCq} method, where $\Delta Cq = Cq_{miRNA} - Cq_{cel\text{-}miR\text{-}39\text{-}3p}$. miRNA levels were log-transformed before being used in statistical analyses.

Statistical analysis. Continuous variables are reported as medians (first quartile − third quartile), while categorical variables are reported as frequencies and percentages. Intergroup comparisons of the baseline characteristics and miRNA levels were performed using the nonparametric Kruskal-Wallis or Mann-Whitney tests for continuous variables and Fisher's

Table 1. Characteristics of the study groups

Variable	Healthy control		idiopathic DCM		Ischemic DCM		*LMNA*^{MUT} carrier		*BAG3*^{MUT} carrier	
	n	Median (Q1-Q3)/n (%)	n	Median (Q1-Q3)/n (%)	N	Median (Q1-Q3)/n (%)	n	Median (Q1-Q3)/n (%)	n	Median (Q1-Q3)/n (%)
Age (years)	70	37.0 (28.0–47.0)	55	68.0 (60.0–75.0)	60	68.0 (62.0–73.0)	37	39.0 (29.5–52.0)	32	43.0 (35.5–52.5)
Sex	70		55		60		37		32	
Male		35 (50.0)		37 (67.3)		48 (80.0)		18 (48.6)		21 (65.6)
Female		35 (50.0)		18 (32.7)		12 (20.0)		19 (51.4)		11 (34.4)
Body mass index (kg/m^2)	70	23.6 (21.1–26.1)	50	28.1 (25.5–30.8)	54	28.7 (25.3–31.3)	37	25.0 (22.8–26.1)	30	27.3 (23.9–30.7)
Hypertension	70	0 (0.0)	55	31 (56.4)	60	46 (76.7)	37	9 (24.3)	32	10 (31.3)
Dyslipidemia	70	0 (0.0)	51	12 (23.5)	59	12 (20.3)	34	5 (14.7)	32	7 (21.9)
Diabetes mellitus	70	0 (0.0)	55	20 (36.4)	59	31 (52.5)	37	4 (10.8)	32	2 (6.3)
Smoking status	70		55		59		37		32	
Never		70 (100.0)		28 (50.9)		28 (47.5)		22 (59.5)		24 (75.0)
Smoker		0 (0.0)		8 (14.5)		7 (11.9)		13 (35.1)		7 (21.9)
Former smoker		0 (0.0)		19 (34.5)		24 (40.7)		2 (5.4)		1 (3.1)
Personal history of CVD	70	0 (0.0)	55	18 (32.7)	60	20 (33.3)	37	18 (48.6)	32	11 (34.4)
Family history of CVD	70	0 (0.0)	55	8 (14.5)	60	7 (11.7)	37	35 (94.6)	32	21 (65.6)
Dilated cardiomyopathy	70	0 (0.0)	55	55 (100.0)	60	60 (100.0)	37	20 (54.1)	32	19 (59.4)
LVEF (%)	70	65.5 (62.0–70.0)	55	36.0 (30.0–42.0)	60	35.0 (31.0–38.8)	37	53.8 (47.8–60.1)	32	46.9 (37.1–56.7)
LVEDD (mm)	70	45.0 (43.0–48.0)	55	60.0 (58.0–64.0)	60	60.0 (57.0–63.0)	37	55.0 (46.5–58.0)	29	56.8 (50.0–62.8)
NYHA functional classes	70		55		60		37		32	
I		70 (100.0)		25 (45.5)		34 (56.7)		24 (64.9)		14 (43.8)
II		0 (0.0)		22 (40.0)		19 (31.7)		10 (27.0)		13 (40.6)
III		0 (0.0)		5 (9.1)		3 (5.0)		3 (8.1)		5 (15.6)
IV		0 (0.0)		3 (5.5)		4 (6.7)		0 (0.0)		0 (0.0)
ICD	70	0 (0.0)	55	8 (14.5)	60	9 (15.0)	36	6 (6.7)	32	3 (9.4)
ACE inhibitor use	70	0 (0.0)	55	1 (1.8)	60	4 (6.7)	37	5 (13.5)	32	0 (0.0)
Beta blocker use	70	0 (0.0)	55	45 (81.8)	60	50 (83.3)	37	5 (13.5)	32	4 (12.5)
Diuretic use	70	0 (0.0)	55	42 (76.4)	60	38 (63.3)	37	3 (8.1)	32	2 (6.3)

Abbreviations: ACE I, angiotensin-converting enzyme; CVD, cardiovascular disease; ICD, implantable cardioverter defibrillator; LVEDD, LV end-diastolic diameter; LVEF, LV ejection fraction.
Data presented as median (Q1–Q3) for continuous variables and frequency (percentage) for categorical variables. Samples size is indicated.

exact test or the chi-square test for categorical variables. Heat map visualizations and cluster analyses were performed based on the miRNA levels.[24] Multiple logistic regression modeling was used to construct the miRNA panels. The regression coefficients of each miRNA significantly associated with the outcome were applied to estimate the miRNA panel value. The diagnostic performance was assessed using receiver operating characteristic (ROC) curve analysis and the derived area under the curve (AUC). An AUC of 0.5 indicates no discrimination above chance, and an AUC of 1.0 indicates perfect discrimination. ROC curves were generated by plotting sensitivity against 1-specificity. The data are presented as the AUC and 95% confidence interval (CI). The comparison of AUCs was performed as proposed by Hanley and McNeil.[25] Logistic regression analyses were used to investigate whether the miRNA panels were independently associated with the outcome. The statistical software R (www.r-project.org) was used for all analyses.

RESULTS

Screening. First, we performed a screen to identify miRNA candidates that are differentially expressed between study groups. We measured the expression of 179 miRNAs previously described in human circulation in plasma samples from 10 idiopathic DCM patients and 20 participants with pathogenic variants associated with DCM: 10 *LMNA*^{MUT} carriers and 10 *BAG3*^{MUT} carriers. Among the *LMNA*^{MUT} and *BAG3*^{MUT} groups, 7 patients were phenotypically positive for DCM (familial DCM). The participants' characteristics are summarized in Supplemental Table S2.

Unsupervised hierarchical clustering of peripheral miRNA levels segregated based on the participant's *LMNA* and *BAG3* variants (wild-type or pathogenic), independently of the presence of the disease (Fig 2). The established criteria for the selection of miRNA candidates were high levels (median Cq<32 and detected in at least 80% of all samples) and statistical significance. There were 22 miRNAs that were found to be highly expressed and differentially detected when comparing i) subjects with pathogenic and wild-type variants in *LMNA* and *BAG3* ($P < 0.1$) and ii) idiopathic and familial DCM ($P < 0.05$) (Supplemental Table S3). The candidates selected for further analysis were let-7g-5p, miR-1-3p, miR-16-5p, miR-16-2-3p, miR-19b-3p, miR-25-3p, miR-29a-3p, miR-30b-5p, miR-30e-3p, miR-130b-3p, miR-133a-3p, miR-133b, miR-150-5p, miR-192-5p, miR-199a-3p, miR-210-3p, miR-215-5p, miR-324-3p, miR-363-3p, miR-532-5p, miR-629-5p, and miR-660-5p. In addition to these candidates, other miRNAs previously reported as biomarkers of familial DCM (let-7a-5p, miR-142-3p, miR-145-5p, and miR-454-3p) were also included in subsequent analyses.[22]

Patients phenotypically positive for DCM. We sought to determine whether the peripheral miRNA pattern could be used diagnostically in patients with DCM. We constructed a miRNA panel to discriminate between patients phenotypically positive for DCM with (*LMNA*^{MUT} and *BAG3*^{MUT}-associated familial DCM) and without (idiopathic and ischemic DCM) pathogenic variants implicated in the development of the disease (Table 2). In univariate analysis, 11 miRNAs differed significantly between both groups: miR-16-5p, miR-16-2-3p, miR-19b-3p, miR-25-3p, miR-130b-3p, miR-133a-3p, miR-210-3p, miR-324-3p, miR-363-3p, miR-629-5p, and miR-660-5p (Supplemental Table S4). As shown in Fig 3A and 3B, we identified a 6-miRNA panel (composed of let-7a-5p, let-7g-5p, miR-16-2-3p, miR-210-3p, miR-215-5p, and miR-629-5p) that discriminated between familial and nonfamilial DCM with an AUC (95% CI) of 87.8 (82.0−93.6). The panel outperformed the best individual miRNA regarding discrimination (miR-210-3p, improvement of 19.7 units in AUC, $P = 0.0002$). The AUC was optimal in the pairwise comparisons between idiopathic or ischemic DCM with *LMNA*^{MUT} or *BAG3*^{MUT}-associated familial DCM (AUCs ranging from 85.9 to 89.9) (Fig 3C). In an attempt to explore the potential clinical application of the miRNA panel, we tested the ability of the signature to rule out familial DCM caused by variants in *LMNA* and *BAG3*. Fig 3D shows the corresponding sensitivity for a whole range of specificities (80%−95%). We also evaluated the distribution of patients with different etiologies across different tertiles of the 6-miRNA panel (Fig 3E). Patients in tertiles 1 and 2 of the distribution of the panel were mainly composed of subjects with idiopathic and ischemic DCM (95/103, 92%). The association of the 6-miRNA panel with familial DCM was independent of demographic, clinical and pharmacological factors (Supplemental Table S5).

Patients phenotypically negative for DCM. We next hypothesized that the peripheral miRNA profile could also be used as a screening tool in the early asymptomatic stages of familial DCM, that is, to identify carriers of pathogenic variants responsible for DCM who are phenotypically negative for the disease. Therefore, we developed a miRNA panel to distinguish between healthy controls with wild-type variants and carriers of pathogenic variants without clinical symptoms or echocardiographic data suggestive of established DCM (Table 3). Significant differences were identified in 16 miRNAs (let-7g-5p, miR-19b-3p, miR-25-3p, miR-29a-3p, miR-130b-3p, miR-133a-3p, miR-145-5p,

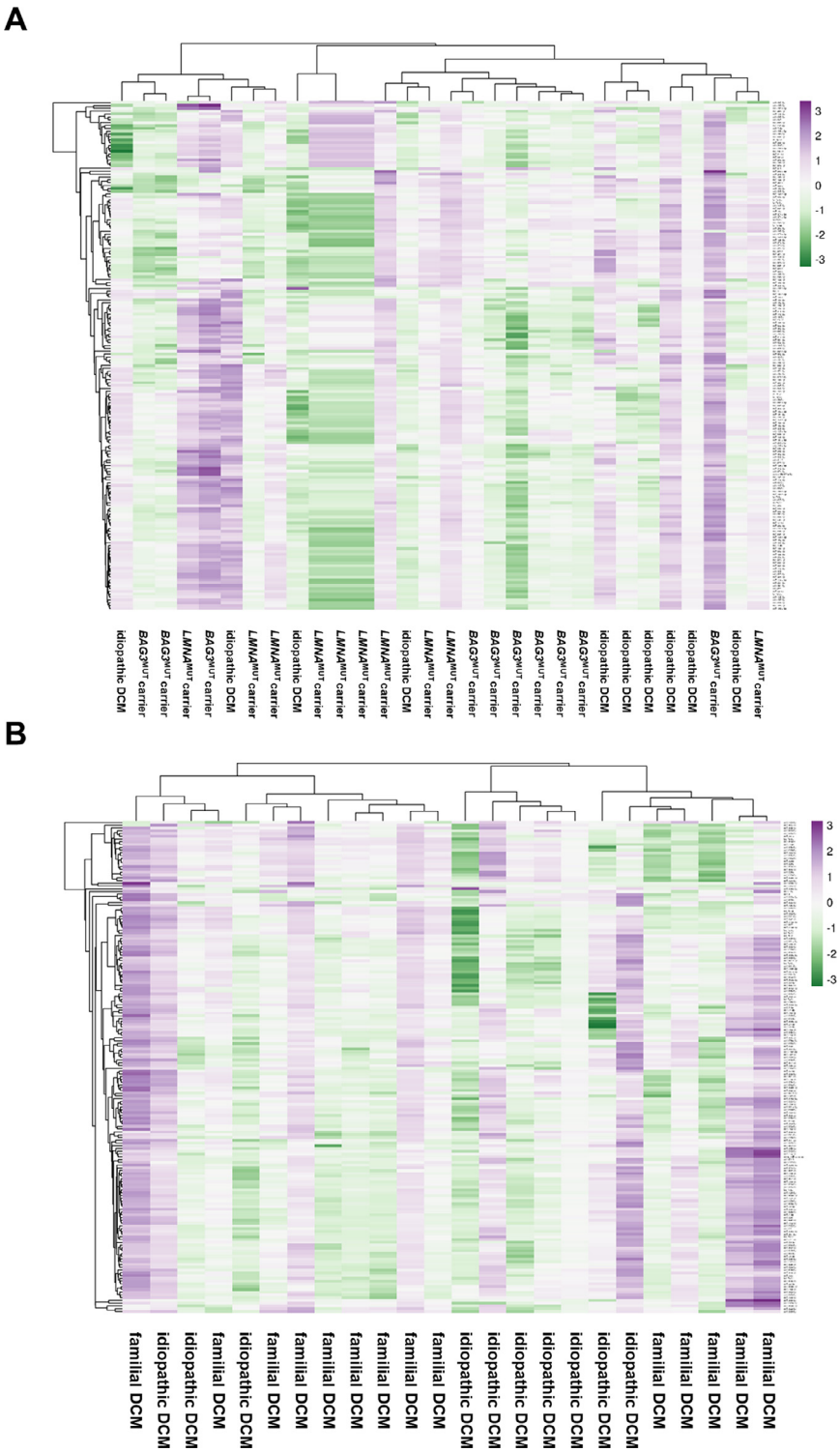

Fig 2. Screening. (A) Peripheral miRNA profile in samples from 10 idiopathic DCM patients and 20 age- and sex-matched participants with pathogenic variants associated with familial DCM: 10 *LMNA*^{MUT} carriers and 10 *BAG3*^{MUT} carriers. (B) Peripheral miRNA profile in samples from 10 idiopathic DCM patients and 14 *LMNA*^{MUT} (n = 7) and *BAG3*^{MUT} (n = 7) carriers phenotypically positive for DCM (familial DCM). The heat map illustrates the levels of peripheral miRNAs. Each column represents a sample. Each row represents a miRNA. Purple spectra represent increasing expression. Green spectra represent decreasing expression.

Table 2. Characteristic of the study groups (phenotypically positive for DCM)

	Nonfamilial DCM (Idiopathic & ischemic DCM)		Familial DCM (*LMNA*^{MUT} & *BAG3*^{MUT} carrier with fDCM)			*LMNA*^{MUT} carrier with fDCM		*BAG3*^{MUT} carrier with fDCM	
Variable	**n**	**Median (Q1–Q3)/n (%)**	**n**	**Median (Q1–Q3)/n (%)**	***P*-value**	**n**	**Median (Q1–Q3)/n (%)**	**n**	**Median (Q1–Q3)/n (%)**
Age (years)	115	68.0 (60.0–74.0)	39	49.0 (39.0–53.0)	<0.001	20	47.0 (39.0–52.8)	19	49.0 (42.0–54.0)
Sex	115		39			20		19	
Male		85 (73.9)		27 (69.2)	0.678		13 (65.0)		14 (73.7)
Female		30 (26.1)		12 (30.8)			7 (35.0)		5 (26.3)
Body mass index (kg/m^2)	104	28.4 (25.3–31.1)	39	26.2 (24.5–29.3)	0.028	20	25.2 (24.3–26.2)	19	27.7 (26.8–31.3)
Hypertension	115	77 (67.0)	39	18 (46.2)	0.024	20	9 (45.0)	19	9 (47.4)
Dyslipidemia	110	24 (21.8)	38	9 (23.7)	0.823	19	3 (15.8)	19	6 (31.6)
Diabetes mellitus	114	51 (44.7)	39	6 (15.4)	0.001	20	4 (20.0)	19	2 (10.5)
Smoking status	114		39		0.001	20		19	
Never		56 (49.1)		24 (61.5)			11 (55.0)		13 (68.4)
Smoker		15 (13.2)		12 (30.8)			7 (35.0)		5 (26.3)
Former smoker		43 (37.7)		3 (7.7)			2 (10.0)		1 (5.3)
Personal history of CVD	115	38 (33.0)	39	17 (43.6)	0.251	20	10 (50.0)	19	7 (36.8)
Family history of CVD	115	15 (13.0)	39	27 (69.2)	<0.001	20	18 (90.0)	19	9 (47.4)
Dilated		cardiomyopathy	115	115 (100.0)	39	39	(100.0)		1.000
20	20 (100.0)	19	19 (100.0)						
LVEF (%)	115	35.0 (30.0–40.0)	39	44.6 (37.0–51.5)	<0.001	20	48.1 (42.7–56.3)	19	41.1 (32.0–45.3)
LVEDD (mm)	115	60.0 (57.0–64.0)	37	59.7 (56.9–63.8)	0.607	20	58.0 (56.2–61.0)	17	62.0 (58.9–64.3)
NYHA functional classes	115		39		0.090	20		19	
I		59 (51.3)		15 (38.5)			11 (55.0)		4 (21.1)
II		41 (35.7)		18 (46.2)			8 (40.0)		10 (52.6)
III		8 (7.0)		6 (15.4)			1 (5.0)		5 (26.3)
IV		7 (6.1)		0 (0.0)			0 (0.0)		0 (0.0)
ICD	115	17 (14.8)	38	4 (10.5)	0.597	19	2 (10.5)	19	2 (10.5)
ACE inhibitor use	115	5 (4.3)	39	4 (10.3)	0.232	20	4 (20.0)	19	0 (0.0)
Beta blocker use	115	95 (82.6)	39	7 (17.9)	<0.001	20	4 (20.0)	19	3 (15.8)
Diuretic use	115	80 (69.6)	39	5 (12.8)	<0.001	20	3 (15.0)	19	2 (10.5)

Abbreviations: ACE I, angiotensin-converting enzyme; CVD, cardiovascular disease; fDCM, familial DCM; ICD, implantable cardioverter defibrillator; LVEDD, LV end-diastolic diameter; LVEF, LV ejection fraction.

Data presented as median (Q1–Q3) for continuous variables and frequency (percentage) for categorical variables. Samples size is indicated. Differences between groups were analyzed using Mann-Whitney test, Fisher's exact test or Chi-square test.

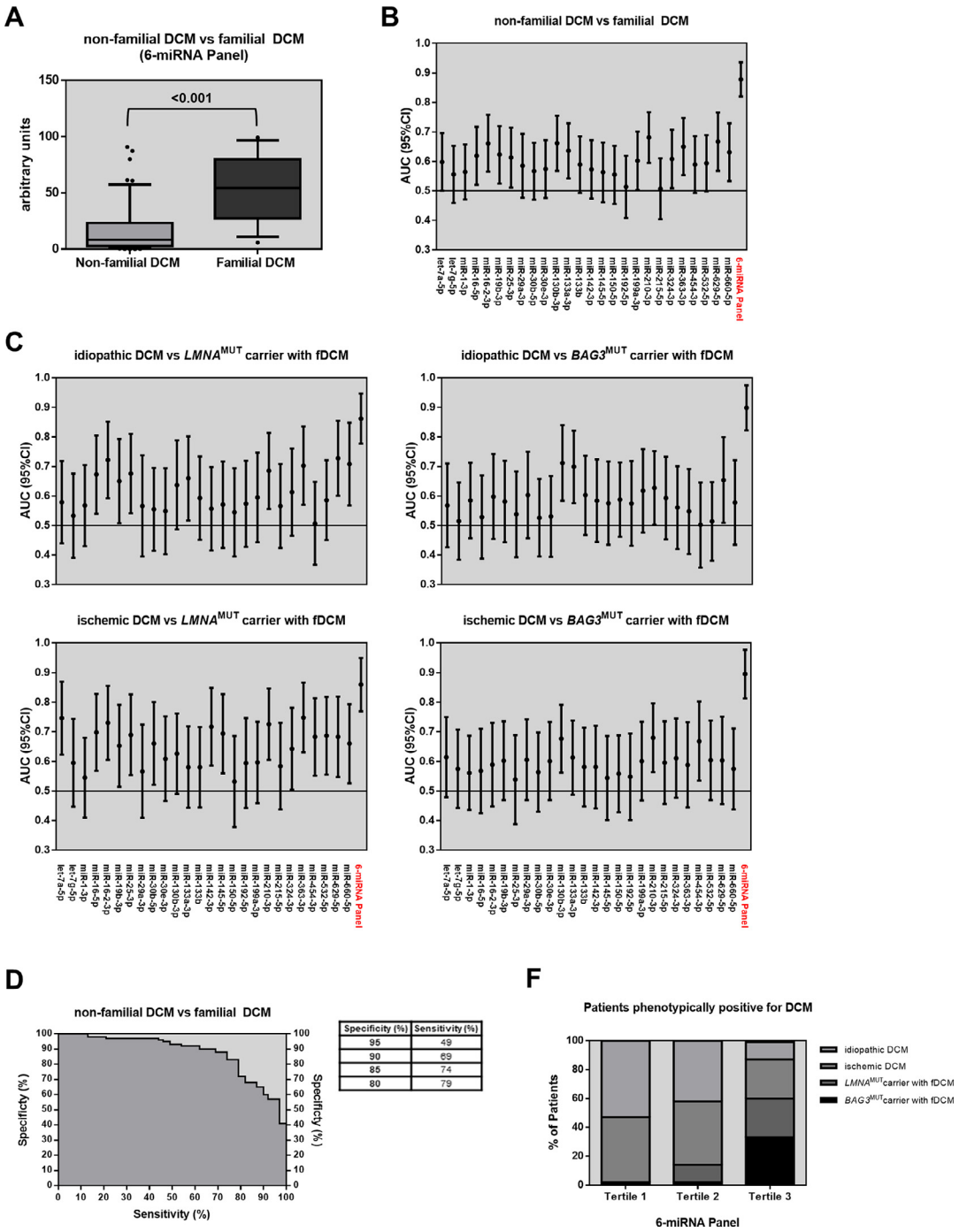

Fig 3. Diagnostic performance of individual miRNAs and the 6-miRNA panel in patients phenotypically positive for DCM. (A) Levels of the 6-miRNA panel in nonfamilial DCM vs familial DCM patients. The data are presented as the median with the 5-95 percentiles. Differences between groups were analyzed using the Mann-Whitney test. (B) Diagnostic performance in nonfamilial DCM vs familial DCM patients. (C) Diagnostic performance in the pairwise comparisons between idiopathic or ischemic DCM with *LMNA*^{MUT} or *BAG3*^{MUT}-associated familial DCM. The diagnostic performance was assessed using receiver operating characteristic (ROC) curve analysis and the derived area under the curve (AUC). The data are presented as the AUC and 95% confidence interval (CI). (D) Corresponding sensitivity for a range of specificities (80%−95%) of the 6-miRNA panel. E) Distribution of patients with different etiologies across tertiles of the 6-miRNA panel. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3. Characteristics of the study groups (phenotypically negative for DCM)

	Healthy control		Carrier without familial DCM (*LMNA*^{MUT} & *BAG3*^{MUT} carrier without DCM)			*LMNA*^{MUT} carrier without DCM		*BAG3*^{MUT} carrier without DCM	
Variable	**n**	**Median (Q1–Q3)/n (%)**	**n**	**Median (Q1–Q3)/n (%)**	***P*-value**	**n**	**Median (Q1–Q3)/n (%)**	**n**	**Median (Q1–Q3)/n (%)**
Age (years)	70	37.0 (28.0–47.0)	30	29.5 (19.0–42.3)	0.047	17	30.0 (23.0–43.0)	13	28.0 (18.5–42.5)
Sex	70		30		0.390	17		13	
Male		35 (50.0)		12 (40.0)			5 (29.4)		7 (53.8)
Female		35 (50.0)		18 (60)			12 (70.6)		6 (46.2)
Body mass index (kg/m^2)	70	23.6 (21.1–26.1)	28	24.8 (21.1–26.6)	0.439	17	24.6 (21.0–25.7)	11	25.1 (21.7–28.4)
Hypertension	70	0 (0.0)	30	1 (3.3)	0.300	17	0 (0.0)	13	1 (7.7)
Dyslipidemia	70	0 (0.0)	28	3 (10.7)	0.022	15	2 (13.3)	13	1 (7.7)
Diabetes mellitus	70	0 (0.0)	30	0 (0.0)	NA	17	0 (0.0)	13	0 (0.0)
Smoking status	70		30		<0.001	17		13	
Never		70 (100.0)		22 (73.3)			11 (64.7)		11 (84.6)
Smoker		0 (0.0)		8 (26.7)			6 (35.3)		2 (15.4)
Former smoker		0 (0.0)		0 (0.0)			0 (0.0)		0 (0.0)
Personal history of CVD	70	0 (0.0)	30	12 (40.0)	<0.001	17	8 (47.19	13	4 (30.8)
Family history of CVD	70	0 (0.0)	30	29 (96.7)	<0.001	17	17 (100.0)	13	12 (92.3)
Dilated cardiomyopathy	70	0 (0.0)	30	0 (0.0)		17	0 (0.0)	13	0 (0.0)
LVEF (%)	70	65.5 (62.0–70.0)	30	57.4 (53.1–60.2)	<0.001	17	59.2 (52.9–61.5)	13	56.2 (53.1–60.0)
LVEDD (mm)	70	45.0 (43.0–48.0)	29	47.0 (43.5–52.4)	0.168	17	46.0 (41.4–52.0)	12	48.3 (44.2–52.5)
NYHA functional classes	70		30		<0.001	17		13	
I		70 (100.0)		23 (76.7)			13 (76.5)		10 (76.9)
II		0 (0.0)		5 (16.7)			2 (11.8)		3 (23.1)
III		0 (0.0)		2 (6.7)			2 (11.8)		0 (0.0)
IV		0 (0.0)		0 (0.0)			0 (0.0)		0 (0.0)
ICD	70	0 (0.0)	30	5 (16.7)	0.009	17	4 (23.5)	13	1 (7.7)
ACE inhibitor use	70	0 (0.0)	30	1 (3.3)	0.300	17	1 (5.9)	13	0 (0.0)
Beta blocker use	70	0 (0.0)	30	2 (6.7)	0.088	17	1 (5.9)	13	1 (7.7)
Diuretic use	70	0 (0.0)	30	0 (0.0)	NA	17	0 (0.0)	13	0 (0.0)

Abbreviations: ACE I, angiotensin-converting enzyme; CVD, cardiovascular disease; fDCM, familial DCM; ICD, implantable cardioverter defibrillator; LVEDD, LV end-diastolic diameter; LVEF, LV ejection fraction; NA, nonapplicable.

Data presented as median (Q1–Q3) for continuous variables and frequency (percentage) for categorical variables. Samples size is indicated. Differences between groups were analyzed using Mann-Whitney test, Fisher's exact test or Chi-square test.

miR-150-5p, miR-192-5p, miR-210-3p, miR-215-5p, miR-324-3p, miR-363-3p, miR-532-5p, miR-629-5p, and miR-660-5p) when comparing both study groups (Supplemental Table S6). We identified a well-discriminating 5-miRNA panel, composed of miR-19b-3p, miR-29a-3p, miR-130b-3p, miR-215-5p and miR-660-5p, that distinguishes between healthy controls and phenotypically negative carriers (AUC [95% CI] = 87.5 [80.4−94.6]) (Fig 4A and 4B). Again, the AUC for the 5-miRNA panel was significantly higher than that for the best individual miRNA (miR-29a-3p, improvement of 14.5 units in AUC, $P = 0.0382$). The AUC was optimal in the pairwise comparisons between healthy controls and *LMNA*^{MUT} or *BAG3*^{MUT} carriers (AUCs ranging from 83.2 to 90.8) (Fig 4C). Additional evaluations were performed to test the biomarker value of the 5-miRNA panel. More specifically, the ability to rule out the presence of pathogenic variants in *LMNA* and *BAG3*. Fig 4D shows the corresponding sensitivity for the range of specificities. As shown in Fig 4E, subjects in tertiles 1 and 2 of the distribution of the 5-miRNA panel were mainly composed of healthy controls (58/67, 87%). The association between the 5-miRNA panel and the presence of pathogenic variants was independent of potential confounders (Supplemental Table S5).

DISCUSSION

The rigorous identification of DCM etiology is of clinical interest in terms of prognostication and the implementation of personalized therapies based on the underlying pathological mechanisms.[26] However, current diagnostic approaches have limitations.[21] The heterogeneous clinical presentation and incomplete penetrance of familial DCM make a correct and timely diagnosis challenging. Once the DCM phenotype is identified using current diagnostic tools, an easily accessible and cost-effective test may be useful to rule-out/rule-in familial DCM, which ultimately could guide clinical decisions in terms of prognostication, family screening and targeted etiology-specific treatments.

Our findings suggest that miRNAs provide value in the management of DCM. We described subsets of peripheral miRNAs that are uniquely expressed in carriers of pathogenic variants responsible for familial DCM. Using a 6-miRNA panel, we were able to discriminate between DCM patients with pathogenic or wild-type variants in *LMNA* or *BAG3* with high accuracy. In more detail, we also discriminated between specific DCM etiologies: ischemic DCM, idiopathic DCM and familial DCM (*LMNA*^{MUT} or *BAG3*^{MUT}). These results are especially relevant in certain clinical scenarios in which the diagnosis is challenging using current clinical tools, for example, discrimination between idiopathic and familial DCM. Our multimarker panel could provide a precise and straightforward approach for initial triage or even differential diagnosis strategies in patients with suspected familial DCM. Indeed, among patients phenotypically positive for DCM in the lowest tertiles of the 6-miRNA panel, only 1 to 10 were patients with familial DCM. The ability of panel to rule out familial DCM was independent of clinical factors. The use of a molecular diagnostic method based on miRNAs could augment the ability to identify patients without the diagnosis, avoiding needless evaluation, reducing patients' uncertainty and anxiety and constraining escalating costs for healthcare systems. In addition, we also developed a 5-miRNA panel for discriminating between healthy controls and *LMNA*^{MUT} and *BAG3*^{MUT} carriers phenotypically negative for the disease. Again, this panel showed a good ability to rule out the presence of pathogenic variants. The lowest tertiles of the 5-miRNA panel were mainly composed of healthy controls (9 to 10). The potential of miRNAs to rule out the presence of pathogenic variants associated with DCM within the generally low-risk population is a novel approach, especially for screening and monitoring the asymptomatic relatives of patients diagnosed with familial DCM.

Multivariable approaches are fundamental when evaluating miRNAs as clinical indicators. Here, the diagnostic power of individual miRNAs was too low to justify their translation to clinical practice. The use of miRNA signatures as biomarkers of DCM has biological sense. First, disease phenotypes are a consequence of abnormalities in entire gene expression networks. Second, coordinated miRNA expression rather than the expression of individual miRNAs is critical for the regulation of complex biological mechanisms. The altered expression of single miRNAs has a modest impact on gene expression and does not recapitulate the complexity of DCM and its etiologies. Supporting previous findings,[27,28] the development of biomarkers based on miRNAs should be focused on panels/signatures more than individual miRNAs. This is especially relevant in the case of *BAG3*^{MUT} carriers for which individual miRNAs showed poor discriminative potential, but the miRNA panels provided useful information in terms of diagnosis. miRNAs play a key role in the development of DCM. The cardiac-specific knockout of Dicer, an endonuclease essential in miRNA biogenesis, leads to DCM.[29] Our miRNA panels may integrate information from molecular pathways associated with the presence of pathogenic variants implicated in inherited DCM, suggesting new targets for intervention. Furthermore, the miRNA signature may provide valuable information about the subclinical mechanisms associated with the

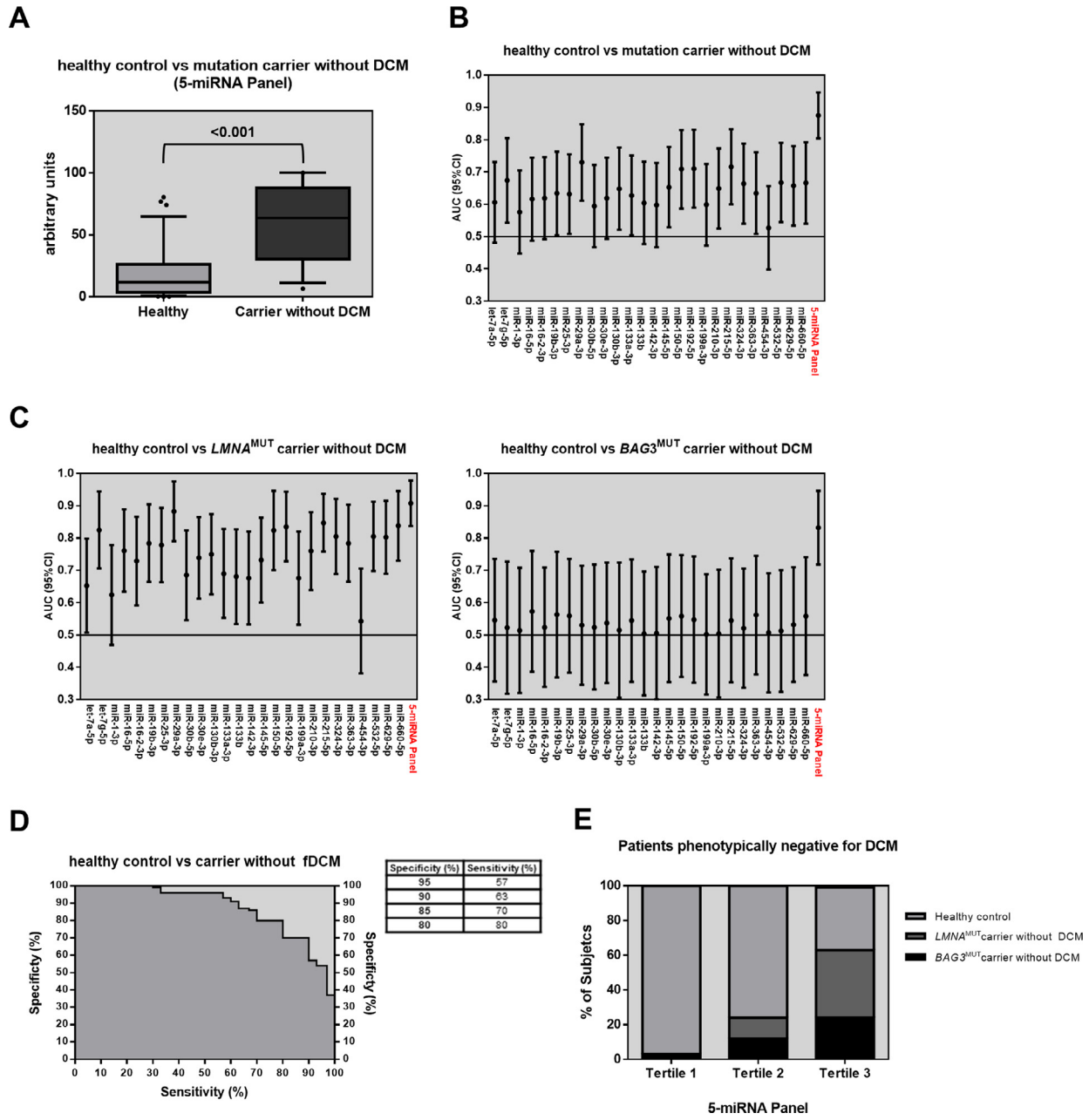

Fig 4. Diagnostic performance of the individual miRNAs and the 5-miRNA panel in subjects phenotypically negative for DCM. (A) Levels of the 5-miRNA panel in healthy controls and carriers of pathogenic variants in *LMNA*^{MUT} or *BAG3*^{MUT} without clinical symptoms or echocardiographic data suggestive of established DCM. The data are presented as the median with the 5−95 percentiles. Differences between groups were analyzed using the Mann-Whitney test. (B) Diagnostic performance in healthy controls and carriers of pathogenic variants in *LMNA*^{MUT} or *BAG3*^{MUT}. (C) Diagnostic performance in the pairwise comparisons between healthy controls with *LMNA*^{MUT} or *BAG3*^{MUT} carriers. The diagnostic performance was assessed using receiver operating characteristic (ROC) curve analysis and the derived area under the curve (AUC). The data are presented as the AUC and 95% confidence interval (CI). (D) Corresponding sensitivity for a range of specificities (80%−95%) of the 5-miRNA panel. (E) Distribution of subjects with different etiologies across tertiles of the 5-miRNA panel. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

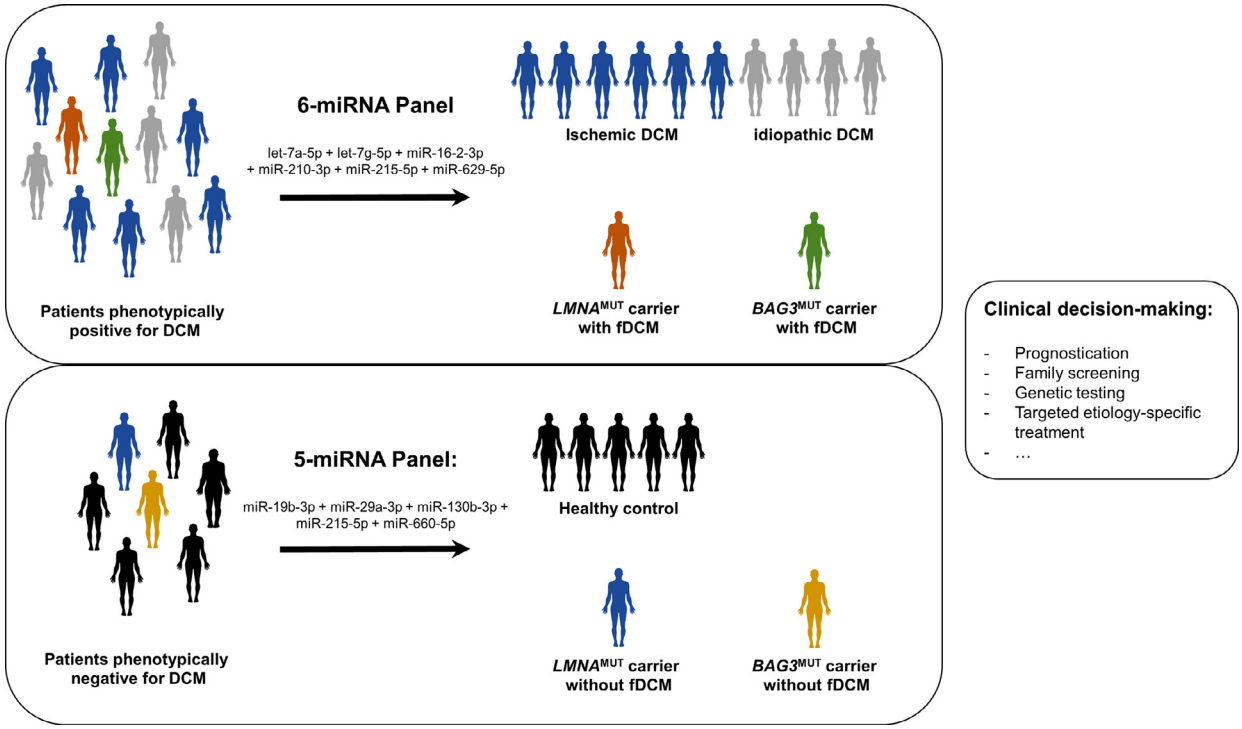

Fig 5. Potential clinical application of the miRNA panels.

development of DCM in patients with pathogenic variants who are phenotypically negative for the disease. However, this discussion should be performed with caution: (1) the observational design precludes causative interpretations; (2) the miRNAs that composed the panels were selected in an automated fashion that limits causal inferences; and (3) despite the promising findings,[30] the origin, transport and fate of peripheral miRNAs and therefore their role as endocrine mediators, is not well understood.[31] Since the extracellular miRNA profile does not completely reflect the intracellular miRNA expression pattern,[32] the identification of the cellular origin is complex. Additionally, the miRNAs that composed the panels have been identified in a wide number of cardiac and noncardiac tissues.[33] The identification of the miRNA transporter (eg extracellular vesicles) and miRNA cargo mechanism may add relevant biological information. However, the use of plasma samples does not allow us to identify the transporter. Nonetheless, from the diagnostic perspective, the miRNA cellular origin or whether the miRNA panels are causally related to the disease are not relevant for the clinical application. A biomarker does not need to guide mechanistic interpretations if it provides useful information in clinical decision-making. In this context, the use of plasma or serum seems to be more appropriate that extracellular vesicles since these sources of miRNAs minimize the preanalytical variation.

CONCLUSIONS

Tests based on peripheral miRNA signatures emerge as a diagnostic method in the medical examinations of DCM. In particular, panels combining miRNAs serve as a robust biomarker to rule out the presence of pathogenic variants associated with familial DCM in both patients phenotypically positive and negative for the disease (Fig 5).

Limitations and strengths. Our conclusions should be interpreted in the context of certain limitations. We acknowledge that the findings need to be confirmed in larger populations. As expected, the study groups composed of patients with pathogenic variants were modestly sized, which limits the comparison of their peripheral miRNA profiles (Supplemental Tables S4 & S6). Additionally, the patients were not consecutively included in the study. This design limits the generalizability of the panels. Future investigations using a "real clinical setting" including unbiased series of patients presenting with initially uncertain diagnoses are needed. Further analyses should also be performed in other *LMNA* and *BAG3* pathogenic variants and other genes associated with familial DCM. For example, the inclusion of patients with pathogenic variants in the *TTN* gene, accounting for approximately 25% of familial cases,[6] seems fundamental. The utility of the panels should also be tested in a cohort of DCM patients without genetic

information available. The panels were constructed in predominantly Caucasian participants. Different ethnic/racial backgrounds should be tested. Nevertheless, the total sample size (n = 254) is the largest sample size used in studies focused on microRNAs, biomarkers, and DCM.[21] The comparison of healthy controls and nonfamilial DCM patients with carriers of pathogenic variants responsible for DCM but phenotypically negative and positive for the disease should be highlighted. This approach limits the overestimation of miRNA accuracy as biological markers.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2020.01.003.

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